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Influence of lipopolysaccharide on proinflammatory gene expression in human corneal, conjunctival and meibomian gland epithelial cells

Di Chen[1,2], **Afsun Sahin**[1,3], **Wendy R. Kam**[1], **Yang Liu**[1], **Raheleh Rahimi Darabad**[1,4], and **David A. Sullivan**[1]

[1]Schepens Eye Research Institute, Massachusetts Eye and Ear, and Department of Ophthalmology, Harvard Medical School, Boston, MA, USA

[2]Department of Ophthalmology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China

[3]Department of Ophthalmology, Koc University Medical School, Istanbul, Turkey

[4]Department of Clinical Anesthesia, Indiana University School of Medicine, Indianapolis, IN, USA

Abstract

Purpose—Lipopolysaccharide (LPS), a bacterial endotoxin, is known to stimulate leuokotriene B4 (LTB4) secretion by human corneal (HCECs), conjunctival (HConjECs) and meibomian gland (HMGECs) epithelial cells. We hypothesize that this LTB4 effect represents an overall induction of proinflammatory gene expression in these cells. Our objective was to test this hypothesis.

Methods—Immortalized HCECs, HConjECs and HMGECs were cultured in the presence or absence of LPS (15 μg/ml) and ligand binding protein (LBP; 150 ng/ml). Cells were then processed for RNA isolation and the analysis of gene expression by using Illumina BeadChips, background subtraction, cubic spline normalization and GeneSifter software.

Results—Our findings show that LPS induces a striking increase in proinflammatory gene expression in HCECs and HConjECs. These cellular reactions are associated with a significant up-regulation of genes associated with inflammatory and immune responses (e.g. IL-1β, IL-8, and tumor necrosis factor), including those related to chemokine and Toll-like receptor signaling pathways, cytokine-cytokine receptor interactions, and chemotaxis. In contrast, with the exception of Toll-like signaling and associated innate immunity pathways, almost no proinflammatory ontologies were upregulated by LPS in HMGECs.

*Corresponding author: Di Chen, MD, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA, USA 02114, Tel: 857-928-6361, di_chen@meei.harvard.edu.

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Conclusions—Our results support our hypothesis that LPS stimulates proinflammatory gene expression in HCECs and HConjECs. However, our findings also show that LPS does not elicit such proinflammatory responses in HMGECs.

Keywords

lipopolysaccharide; inflammation; gene expression; ocular surface epithelial cells

Introduction

The human ocular surface is inhabited with diversified commensal microorganisms, many of which are bacteria. These organisms account for more than 90% of the eye's surface microbiota, and possess both potent immunoregulatory functions and pathogenic capabilities [1–3]. Indeed, bacteria and their toxins can cause significant, and sometimes irreversible, damage to the ocular surface [4, 5]. For example, bacterial keratitis accounts for 41.8% to 91.8% of all corneal infections, and is one of the most frequent causes of corneal blindness [6–8]. Further, bacterial conjunctivitis is the major cause of red eye worldwide, [9] and bacterial toxins may contribute to the development of obstructed terminal ducts in meibomian gland dysfunction (MGD) [10]. However, the mechanism(s) that underlie the diverse array of these bacterial actions on ocular surface and adnexal epithelial cells have yet to be elucidated.

We hypothesize that one such bacterial mechanism involves a lipopolysaccharide (LPS)-induced proinflammatory gene expression in these epithelial cells. LPS, also known as endotoxin, is a glycolipid of Gram-negative bacteria cell walls that elicits strong inflammatory responses in mammalian cells[11]. LPS is composed of a hydrophilic polysaccharide and a hydrophobic lipid A, which is the toxic component of LPS and activates the host's innate immune system via the Toll-like recepter4/myeloid differentiation factor 2 (TLR4/MD2) receptor complex[12–14]. The lipid transferase LPS-binding protein (LBP) and CD14 catalyze LPS transfer to the TLR4/MD2 complex[14].

In support of our hypothesis, we have previously shown that LPS stimulates leukotriene B4 (LTB4) secretion by human corneal (HCEC), conjunctival (HConjEC) and HMG (HMGEC) epithelial cells, and that this effect is enhanced by co-exposure to LBP[15]. To extend these findings, we examined whether LPS and LBP upregulate the expression of numerous inflammatory pathways in these cells.

Methods

Cell cultures

Immortalized HCEC (from Dr. James Jester, Irvine, CA) [16], HConjEC (from Dr. Ilene Gipson, Boston, MA) [17], and HMGEC [18]were cultured, as previously described.[15] In brief, HCEC and HConjEC were cultured in keratinocyte serum free medium (KSFM) supplemented with bovine pituitary extract (BPE, 25 μg/ml), epidermal growth factor (EGF, 50 ng/ml), penicillin and streptomycin. HMGEC were cultured in KSFM supplemented with 50 μg/ml BPE, 50 ng/ml EGF, penicillin, and streptomycin. Cells were maintained in 75 cm^2

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flasks and then plated for experiments in 6-well culture dishes (Corning, Lowell, MA). At confluence the cell numbers ranged from 3.8 to 4.9 x 10^5 cells/well, and varied depending upon the cell type. All cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA), except for DMEM/F12, which was obtained from Mediatech, Inc. (Manassas, VA).

After reaching confluence, cells were rinsed with PBS and then cultured in a medium containing DMEM/F12 with 10% FBS, 10 ng/ml EGF, penicillin and streptomycin for 2 days. After this time period, cells (n = 3 wells/cell type/treatment) were incubated in serum-free DMEM/F12 and exposed to vehicle (1% bovine serum albumin [BSA]; Sigma-Aldrich), or LPS (15 μg/ml; E. Coli, strain 0127:B8, lot #050M4094; Sigma-Aldrich, St. Louis, MO) and LBP (150 ng/ml; R&D Systems, Inc. Minneapolis, MN), for six hours. The LPS and LBP were dissolved in DMEM and the BSA in PBS (Mediatech, Inc., Manassas, VA). The LPS+LBP concentrations used in these studies, as we previously reported [15], were very effective for the stimulation of LTB4 production by ocular surface and adnexal cells.

RNA extraction and gene microarray analysis

Cellular RNA samples were processed for microarray analyses, as previously reported. [19]Briefly, total RNA was extracted using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's instructions. The RNA concentrations and 260/280 nm ratios were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA integrity was evaluated by using a RNA Nano 6000 Series II Chip with a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The RNA samples were further processed by Asuragen (Austin, TX) for quantitation of mRNA levels using microarray expression analysis (HumanHT-12 v.4 Expression BeadChips; Illumina, San Diego, CA), as previously described. [20]

Non-log-transformed, background subtracted and cubic spline normalized data were analyzed with commercial software (GeneSifter.net; Geospiza, Seattle, WA). This comprehensive program also generated gene KEGG pathway, ontology and z-score reports. Standardized hybridization intensity values were adjusted by adding a constant, such that the lowest intensity value for a sample equaled 16.[21, 22] BeadChip data were analyzed with Student's t-test (two-tailed, unpaired). All data are accessible for download through the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) via series accession number GSE111496.

Results

LPS+LBP influence on overall gene expression in human ocular surface and adnexal epithelial cells

To determine endotoxin's influence on gene expression in HCECs, HConjECs and HMGECs, we exposed differentiated cells (n = 3 wells/cell type/treatment) to vehicle or LPS +LBP for six hours and then processed samples for Illumina BeadChip and Geospiza software analyses.

Our results demonstrate that LPS+LBP exert a significant effect on the expression of more than 1,000 genes in HCECs, HConjECs and HMGECs (Table 1). The relative direction of

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this endotoxin impact was similar in all three cell types, with LPS+LBP increasing and decreasing almost the same percentages of genes (i.e. cornea: 52.5% ↑; conjunctiva: 42.4% ↑; meibomian: 44.3% ↑). Some of the most highly up- and down-regulated genes in HCECs, HConjECs and HMGECs following LPS+LBP exposure are shown in Table 2.

There were 9 genes that were upregulated, and 12 genes that were downregulated, in all three cell lines. Examples (with accession numbers) of genes significantly ($p < 0.05$) stimulated by LPS+LBP in all three cell types included those encoding β2-adrenergic receptor (NM_000024), angiopoietin-4 (NM_139314), interleukin-1 (IL-1) receptor-associated kinase 2 (IRAK2; NM_001570), IL-1 receptor antagonist (RA; NM_173843), IL-1α (NM_000575), IL-1β (NM_000576) superoxide dismutase 2 (NM_00102446) and thioredoxin reductase 1 (NM_001093771). Genes significantly ($p < 0.05$) downregulated by LPS+LBP in HCECs, HConjECs and HMGECs included EGF-containing fibulin-like extracellular matrix protein 1 (NM_001039348), G protein-coupled estrogen receptor 1 (NM_001039966), Rab7B (NM_177403), serine protease 23 (NM_007173) and signal transducer and activator of transcription 1 (NM_007315).

Impact of LPS+LBP exposure on pro-inflammatory gene expression in HCECs

Exposure of HCECs to LPS+LBP induced a significant increase in the expression of proinflammatory genes. In fact, of the 20 genes with known functions that were most highly upregulated by LPS+LBP in HCECs, 17 were linked to inflammation (Table 3). This effect was associated with a marked rise in the activity of KEGG pathways mediating a diverse array of inflammatory and immune responses, including cytokine-cytokine receptor interactions and the signaling of Toll-like (TLR), B cell and T cell receptors (Table 4).

Most striking was the influence of LPS+LBP on inflammatory response gene ontologies in HCECs. The endotoxin significantly increased the expression of 52 inflammatory response ontologies with a z-score ≥ 4.0, and another 66 inflammatory response ontologies with z-scores between 2.0 to 4.0. Indeed, 17.7% of the 665 biological process ontologies up-regulated by LPS+LBP were immune-related. Examples of the inflammatory response ontologies stimulated by LPS+LBP in HCECs are shown in Table 5, and include those associated with immune system processes, lymphocyte activation and chemokine production.

Effect of LPS+LBP treatment on pro-inflammatory gene expression in HConjECs

Treatment of HConjECs with LPS+LBP stimulated a significant increase in the activity of numerous pro-inflammatory genes. Of the 25 genes with known functions that were most highly upregulated by LPS+LBP in HConjECs, 15 were linked inflammation (Table 6). This immune response of HConjECs to LPS+LBP was associated with a significantly enhanced expression of inflammatory KEGG pathways, such as those mediating cytokine-, chemokine- and TLR signaling (Table 7).

As we also observed in HCECs, the most impressive effect of LPS+LBP on HConjECs was the up-regulation of inflammatory response gene ontologies. The endotoxin significantly increased the expression of 54 inflammatory response ontologies with a z-score ≥ 4.0, and another 85 inflammatory response ontologies with z-scores between 2.0 to 4.0. Of the 587

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biological process ontologies significantly stimulated by LPS+LBP in HConjECs, 23.7% were immune-associated. Examples of the inflammatory response ontologies up-regulated by LPS+LBP in HConjECs are listed in Table 8, and include those linked to immune system processes, and the chemotaxis, migration and adhesion of leukocytes.

Influence of LPS+LBP exposure on pro-inflammatory gene expression in HMGECs

In contrast to the responses of HCECs and HConjECs, exposure of HMGECs to LPS+LBP did not induce a dramatic increase in the activity of pro-inflammatory genes. Of the 25 genes with known function that were most highly upregulated by LPS+LBP in HMGECs, only 2 were immune-related (Table 10). In addition, the extent to which endotoxin stimulated any immune-linked gene expression in HMGECs was typically less than 1.3-fold, as compared to vehicle (Table 11).

LPS+LBP elicited very few significant differences in KEGG pathways associated with the immune system in HMGECs (Table 12). There were also no inflammatory response gene ontologies upregulated by LPS+LBP in HMGECs. Rather, almost all of the immune-related ontologies that were significantly increased were those related to endotoxin-induced TLR signaling and the associated innate immune and type 1 interferon (IFN) gene activities (Table 13). The significance of these ontological responses was also far less than found in HCECs and HConjECs. There were no LPS+LBP-induced immune ontologies in HMGECs with z-scores greater that 5.0, only 7 such ontologies with a z-score higher than 4.0, and only 21 immune ontologies with a z-score between 2.0 to 4.0. Overall, LPS+LBP significantly increased only 268 biological process ontologies in HMGECs, and of these, 10.4% were linked to the immune system.

Discussion

Our results support our hypothesis that LPS+LBP stimulates proinflammatory gene activity in HCECs and HConjECs. This endotoxin significantly increased the expression of 118 inflammatory response gene ontologies in HCECs, and 139 such ontologies in HConjECs. In contrast, and not in support of our hypothesis, LPS+LBP exposure did not induce a marked increase in the activity of pro-inflammatory genes in HMGECs. Indeed, there were also no inflammatory response gene ontologies upregulated by LPS+LBP in HMGECs. These findings suggest that, unlike HCECs and HConjECs, HMGECs are resistant to the endotoxin-induced stimulation of proinflammatory pathways.

We discovered that LPS+LBP significantly increased the expression of more than 1,000 genes in each of the ocular cell lines. However, many of these genes were cell-specific, and only 21 genes were regulated in the same way in HCECs, HConjECs and HMGECs. The nature of many of these changes was analogous to that found in other cells and tissues. For example, LPS has also been shown to increase the expression of angiopoietin-4, IRAK2, superoxide dismutase 2, thioredoxin reductase 1, IL-1RA, IL-1α and IL-1β in heart, muscle and adipose tissue[23], macrophages [24–26], monocytes [27], microglia [28] and the brain[29]. Similarly, LPS is known to downregulate the expression serine proteases and Rab7B in macrophages [30] and monocytes[31].

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Most impressive was the striking LPS+LBP-induced in increase in proinflammatory gene expression in HCECs and HConjECs. These cellular reactions were associated with a significant upregulation of genes linked to inflammatory and immune responses, including those related to chemokine and Toll-like receptor signaling pathways and cytokine-cytokine receptor interactions. Some of the most highly upregulated genes by LPS+LBP in HCECs and HConjECs included chemokine (C-C motif) ligand 20, which attracts dendritic cells and effector/memory T- and B-cells; IL-1 family, member 9, which activates NF-kappa-B and MAPK signaling pathways in target cells; chemokine (C-X-C motif) ligand 10, which recruits monocytes and T-lymphocytes; lymphotoxin β, which induces the inflammatory response system; TNFα, which plays a major role in inflammation; nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, which encodes a subunit of the transcription factor complex nuclear factor-kappa-B. The NFkappaB complex functions as a central activator of genes involved in inflammation and immune function; IL-8, which is a chemotactic factor that attracts neutrophils, basophils, and T-cells, and is involved in neutrophil activation; prostaglandin-endoperoxide synthase 2, which is responsible for production of inflammatory prostaglandins; and IRAK2, which is reported to participate in the IL1-induced upregulation of NF-kappaB[32, 33].

Even more impressive was that exposure of HMGECs to LPS+LBP did not induce a dramatic increase in the activity of proinflammatory genes. Rather, the immune response of HMGECs to this endotoxin appeared muted, and involved the upregulation of almost no inflammatory response gene ontologies. Instead, those immune-related ontologies that were significantly increased were related primarily to endotoxin-induced TLR signaling and the associated innate immune and type I IFN gene activities. This response might be expected, given that LPS binds to TLRs to stimulate innate immunity, and induce type I IFN production to help protect against bacterial infection[34, 35].

There are several possible explanations for this lack of an HMGEC inflammatory response to LPS+LBP. First, we discovered that the most highly expressed gene in the HMG encodes for leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) [20]. LAIR-1 is an inhibitory receptor that suppresses immune cell activation and attenuates proinflammatory cytokine production [36, 37]. Second, we have found that expression of the LAIR-1 gene is upregulated during HMGEC differentiation. [38] Third, we have discovered that HMGEC differentiation also increases the gene expression for secretoglobin, family 1A, member 1 (i.e. uteroglobin), which suppresses inflammation.[39]

In addition to these anti-inflammatory activities, the MG also features anti-infective properties. We found that HMGEC differentiation is associated with an upregulation of the genes for phospholipase A2, which kills gram-positive bacteria [40], and CCL28, which has broad-spectrum antimicrobial activity [41]. [38] We also learned that human MGD is accompanied by a significant increase in intraglandular transcripts for S100 calcium binding proteins A8 and A9 (S100A8/9, also called calprotectin) [20]. In high concentrations this heterodimer has anti-inflammatory and anti-microbial functions and makes epithelial cells more resistant to bacterial invasion [42–45]. Transcripts for peptidase inhibitor 3, skin-derived (also called elafin) [46]), a bacterial infection inhibitor[33], and S100A7 (also called psoriasin), an antimicrobial peptide[33], are also increased in MGD[20]. Furthermore, we

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have discovered that HMGEC lysates inhibit the growth rate of the gram-negative bacteria, *Pseudomonas aeruginosa* [47], and others have shown that HMGEC lipids prevent the growth of both gram-positive and gram-negative bacteria[48].

Overall, this apparent resistance of the HMG to inflammation and infection may help to explain why there is no evidence of inflammation or infection in this tissue in obstructive MGD [49–53]. These findings do not mean that HMGs cannot become inflamed or infected. A single MG, for example, may develop a chalazion (i.e. inflammation of a blocked gland), that may become secondarily infected. In addition, we have discovered that that isotretinoin can induce the expression of inflammatory mediators in HMGECs[19]. However, neither inflammation nor infection is a characteristic of MGD, which affects multiple glands[54].

Our study has some limitations. First, our data originate from the use of Illumina BeadChips, and as we have previously reported, significant differences may exist between microarray platforms in their ability to detect differential gene expression[55, 56]. This low concordance in gene identification seems to be due to innate differences in platform design, including variations in probe length and content, deposition technology, labeling approaches, hybridizing protocols, image segmentation, signal detection, background correction, data standardization and data mining[21, 57–59]. However, most gene expression changes revealed by a given platform are believed to be biologically correct, and these differences cannot be attributed to technological variations[57, 58]. Nevertheless, it is possible that additional differentially-expressed genes might be identified by using another type of microarray platform. Second, microarray gene expression analysis only reflects the transcript level of genes. We have yet to determine whether these gene transcripts are translated into functional proteins. Third, only immortalized cells were treated and analyzed in our study. There may be more complex interactions *in vivo* and appropriate animal models may be needed to conform our observations.

In summary, our results support our hypothesis that LPS+LBP stimulates proinflammatory gene expression in HCECs and HConjECs. However, our findings also show that LPS+LBP does not elicit such proinflammatory responses in HMGECs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Influence of LPS+LBP exposure on gene expression in HCECs, HConjECs and HMGECs

Immortalized Human Epithelial	LPS+LBP	Vehicle >	Total
Cell Type	> Vehicle	LPS+LBP	genes
Cornea	563	510	1,073
Conjunctiva	786	1,068	1,854
Meibomian gland	613	771	1,384

Data were evaluated without log transformation. The expression of listed genes was significantly ($p < 0.05$) up regulated in cells exposed to LPS +LBP or vehicle treatment.

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Table 2

Impact of LPS+LBP treatment on the expression of selected genes in HCECs, HConjECs and HMGECs

Gene accession #	Gene	Cell	Ratio, p value	Protein function
NM_004591	Chemokine (C- C motif) ligand 20	HCEC	25.2 ↑, < 0.0005	Acts as a ligand for C-C chemokine receptor CCR6. Signals through binding and activation of CCR6 and is responsible for the chemotaxis of dendritic cells, effector/memory T-cells and B-cells
NM_020717	Shroom family member 4	HCEC	6.55 ↓, $p < 0.05$	May regulate cellular and cytoskeletal architecture
NM_054031	MAS related GPR family member X3	HConjEC	2.54 ↑, $p < 0.01$	Serves as a member of the mas-related/sensory neuron specific subfamily of G protein coupled receptors, and may be involved in sensory neuron regulation and modulation of pain
NM_176095	CDK5 regulatory subunit associated protein 3	HConjEC	2.20 ↓, $p < 0.01$	Acts in signaling pathways governing transcriptional regulation and cell cycle progression
NM_139314	Angiopoietin-like 4	HMGEC	2.43 ↑, $p < 0.05$	Regulates glucose homeostasis, lipid metabolism, and insulin sensitivity
NM_002655	PLAG1 zinc finger	HMGEC	3.11 ↓, $p < 0.005$	Transcription factor activation results in up-regulation of target genes, such as insulin-like growth factor 2, leading to uncontrolled cell proliferation

Some of the genes that were highly up- or down-regulated by LPS+LBP are shown. Relative ratios were calculated by comparing the degree of gene expression in cells exposed to vehicle or LPS+LBP. The mean gene intensity level in at least one group exceeded 70 BeadChip units. The source of the protein functions of genes, if transcribed, is: - http://www.genecards.org

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Table 3

LPS+LBP upregulation of inflammatory gene expression in HCECs

Accession number	Gene	Ratio	P value
NM_004591	Chemokine (C-C motif) ligand 20	25.19	0.0003
NM_019618	Interleukin (IL)-1 family, member 9	19.11	0.0000
NM_001165	Baculoviral IAP repeat-containing 3	16.9	0.0044
NM_002341	Lymphotoxin β	11.63	0.0007
NM_001565	Chemokine (C-X-C motif) ligand 10	10.61	0.0000
NM_000594	Tumor necrosis factor α (TNF α)	7.9	0.0033
NM_006509	Transcription factor RelB	7.88	0.0204
NM_006290	TNF α-induced protein 3	7.1	0.0007
NM_006291	TNF α-induced protein 2	6.89	0.0010
NM_025079	Zinc finger CCCH-type containing 12A	5.09	0.0066
NM_001077493	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	4.69	0.0015
NM_000584	IL-8	4.24	0.0099
NM_176823	S100 calcium binding protein A7A	4.23	0.0106
NM_000963	Prostaglandin-endoperoxide synthase 2	4.09	0.0002
NM_001570	IL-1 receptor-associated kinase 2	4.06	0.0001
NM_000710	Bradykinin receptor B1	3.71	0.0194
NM_001511	Chemokine (C-X-C motif) ligand 1	3.25	0.0105

These genes are 17 of the 20 most highly upregulated genes with known functions by LPS+LBP, and all 17 genes are associated with inflammation. Relative ratios were calculated by comparing the degree of gene expression in HCECs treated with LPS+LBP, relative to vehicle.

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Table 4

LPS+LBP impact on KEGG pathways in HCECs

KEGG Pathway	LPS+LBP Genes ↑	Vehicle Genes ↑	LPS+LBP z- score	Vehicle z- score
Apoptosis	13	4	6.57	1.38
NOD-like receptor signaling pathway	9	0	5.38	−1.23
Cytokine-cytokine receptor interaction	17	6	3.25	−0.16
Graft-versus-host disease	4	0	2.96	−0.92
Toll-like receptor signaling pathway	8	3	2.92	0.4
B cell receptor signaling pathway	6	2	2.58	0.19
T cell receptor signaling pathway	7	2	2.17	−0.34
JAK-STAT signaling pathway	9	4	2.13	0.21
Fc gamma R-mediated phagocytosis	6	2	2.08	−0.08
Epithelial cell signaling in Helicobacter pylori infection	5	0	2.14	−1.28
Allograft rejection	3	0	2.08	−0.89

KEGG pathways were selected after the analysis of non-log-transformed Illumina BeadChip data. The criterion for inclusion was a pathway linked to inflammation and a z-score > 2.0. A z-score is a statistical rating of the relative expression of genes, and demonstrates how much the genes are over- or under-represented in a given list. [22] Positive z scores reflect a greater number of genes meeting the criterion than is expected by chance, whereas negative z scores reflect fewer genes meeting the

criterion than expected by chance. [22] Z-scores with values > 2.0 and < −2.0 are quite significant. Terms: LPS+LBP Genes ↑- number of genes up-regulated in LPS+LBP -treated cells; Vehicle Genes ↑- number of genes up-regulated vehicle-treated cells; z-score - specific score for the upregulated genes in the LPS+LBP - and vehicle-treated HCECs.

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Table 5

Influence of LPS+LBP on the expression of inflammatory response gene ontologies in HCECs

Biological process ontology	LPS+LBP Genes ↑	Vehicle Genes ↑	LPS+LBP z-score	Vehicle z- score
Inflammatory response	32	8	7.22	−0.02
Lipopolysaccharide-mediated signaling pathway	6	1	7.19	0.77
Hemopoietic or lymphoid organ development	31	8	7	0.03
Myeloid leukocyte differentiation	13	3	6.92	0.75
Immune system development	32	9	6.8	0.15
Immune system process	75	24	6.77	−1.12
Leukocyte differentiation	21	4	6.52	−0.31
Leukocyte activation	31	8	6.22	−0.32
Cell activation involved in immune response	11	2	5.93	0.13
Leukocyte activation involved in immune response	11	2	5.93	0.13
α- β T cell differentiation	7	0	5.77	−0.95
Regulation of chemokine production	6	2	5.74	1.63
Defense response	50	13	5.54	−1.55
Regulation of adaptive immune response	9	0	5.5	−1.22
Chemokine production	6	2	5.5	1.52
Immune response	49	16	5.4	−0.8
Lymphocyte activation	25	5	5.21	−0.98
T cell activation involved in immune response	5	0	5.11	−0.78

Designated inflammatory response ontologies from the biological process category were selected after the analyses of non-log-transformed data. Criteria for inclusion were an ontology containing ≥ 5 genes and having a z-score > 5.0.

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Table 6

LPS+LBP stimulation inflammatory gene expression in HConjECs

Accession number	Gene	Ratio	P value
NM_000759	Colony stimulating factor 3 (granulocyte)	2.36	0.0004
NM_002438	Mannose receptor, C type 1	2.23	0.0322
NM_007115	TNF α-induced protein 6	2.05	0.0015
NM_013278	IL-17C	1.94	0.0109
NM_000758	Colony stimulating factor 2 (granulocyte- macrophage)	1.93	0.0021
NM_019618	IL-1 family, member 9	1.88	0.0003
NM_016584	IL-23, α subunit p19	1.81	0.0142
NM_001001437	Chemokine (C-C motif) ligand 3-like 3	1.79	0.0010
NM_000201	Intercellular adhesion molecule 1	1.72	0.0003
NM_005098	Musculin	1.72	0.0235
NM_198845	Sialic acid binding Ig-like lectin 6	1.69	0.0027
NM_002341	Lymphotoxin α	1.67	0.0019
NM_000634	IL-8 receptor, α	1.65	0.0333
NM_012323	Transcription factor MafF	1.64	0.0147
NM_006850	IL-24	1.63	0.0048

These genes are 15 of the 25 most highly upregulated genes with known functions by LPS+LBP, and all 15 genes are linked to inflammation. Relative ratios were calculated by comparing the degree of gene expression in HConjECs treated with LPS+LBP, relative to vehicle.

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Table 7

LPS+LBP effect on KEGG pathways in HConjECs

KEGG Pathway	LPS+LBP Genes ↑	Vehicle Genes ↑	LPS+LBP z-score	Vehicle z- score
NOD-like receptor signaling pathway	10	2	5.04	−0.69
Chemokine signaling pathway	18	7	4.16	−0.89
Cytokine-cytokine receptor interaction	21	10	3.36	−1.14
Toll-like receptor signaling pathway	10	6	3.14	0.33
Hematopoietic cell lineage	8	3	2.75	−0.64
Neurotrophin signaling pathway	11	11	2.84	1.81
Epithelial cell signaling in Helicobacter pylori infection	6	3	2.14	−0.28
Graft-versus-host disease	4	2	2.33	0.15
Apoptosis	7	6	2.01	0.71

KEGG pathways were chosen after the analysis of non-log-transformed Illumina BeadChip data in HConjECs. The criterion for inclusion was a pathway associated with inflammation and a z-score > 2.0.

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Table 8

Effect of LPS+LBP on the expression of inflammatory response gene ontologies in HConjECs

Biological process ontology	LPS+LBP Genes ↑	Vehicle Genes ↑	LPS+LBP z-score	Vehicle z- score
Cellular response to interleukin-1	5	1	6.83	0.31
Inflammatory response	34	22	6.75	0.74
Positive regulation of defense response	20	7	6.53	-0.56
Regulation of JAK-STAT cascade	9	1	6.52	-0.87
Leukocyte chemotaxis	12	1	6.47	-1.47
Defense response	61	55	6.45	1.42
Regulation of acute inflammatory response	7	1	6.18	-0.48
Immune response	59	54	6.14	1.34
Positive regulation of interleukin-6 production	6	0	5.99	-1.16
Immune system process	79	85	5.85	1.96
Response to cytokine stimulus	28	21	5.8	1.2
Cell chemotaxis	12	1	5.74	−1.67
Response to interleukin-1	7	1	5.68	−0.62
Cellular response to lipopolysaccharide	8	2	5.57	−0.23
Neutrophil chemotaxis	6	0	5.41	−1.25
Acute inflammatory response	11	6	5.39	0.94
Regulation of interleukin-6 production	8	2	5.39	−0.29
Leukocyte migration	19	10	5.3	0.01
Positive regulation of cytokine production	15	4	5.29	−1.16
Lipopolysaccharide-mediated signaling pathway	5	1	5.29	−0.11
Leukocyte cell-cell adhesion	6	2	5.28	0.38
Innate immune response	31	28	5.22	1.63
Interleukin-6 production	8	2	5.22	−0.35
Positive regulation of inflammatory response	8	1	5.22	−1
Regulation of inflammatory response	13	6	5.14	0.12
Cellular response to cytokine stimulus	21	15	5.08	0.86
Positive regulation of innate immune response	14	6	5.04	−0.23

Designated inflammatory response ontologies from the biological process category were chosen after the analyses of non-log-transformed data. Criteria for inclusion were an ontology containing ≥ 5 genes and having a z-score > 5.0.

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Table 9

Comparative percentage increase in gene expression following LPS+LBP treatment of HCECs, HConjECs and HMGECs

Gene	HCEC	HConjEC	HMGEC
Chemokine (C-C motif) ligand 20	2,519	151	-
IL-1 family, member 9	1,911	188	-
Lymphotoxin β	1,163	167	-
Chemokine (C-X-C motif) ligand 10	1,061	148	(157)
TNF α	790	129	-
TNF α-induced protein 3	710	141	-
Zinc finger CCCH-type containing 12A	509	123	121
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	469	145	122
IL-8	424	127	-
S100 calcium binding protein A7A	423	147	-
Prostaglandin-endoperoxide synthase 2	409	137	-
IL-1 receptor-associated kinase 2	406	118	126
IL-1α	268	125	121
IL-1β	224	117	127

Pecentages were calcualted by comparing the degree of gene expression in cells exposed to LPS+LBP, relative to vehicle. The value in parenthesis represents downregulation.

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Table 10

LPS+LBP upregulation of HMGEC genes

Accession number	Gene	Ratio	P value
NM_139314	Angiopoietin-like 4	2.43	0.0131
NM_201564	Synaptonemal complex central element protein 1	2.11	0.0108
NM_032250	Ankyrin repeat domain 20 family, member A1	2.01	0.0437
NM_178493	Notum pectinacetylesterase homolog	2	0.0226
XM_166227	Macrophage expressed 1	1.96	0.0250
NM_052923	SCAN domain containing 3	1.77	0.0073
NR_002174	Cytidine monophosphate-N-acetylneuraminic acid hydroxylase pseudogene	1.76	0.0008
NM_000132	Coagulation factor VIII, procoagulant component	1.74	0.0293
NM_006798	UDP glucuronosyltransferase 2 family, polypeptide A1	1.66	0.0406
NM_003278	C-type lectin domain family 3, member B	1.64	0.0058
NM_138764	BCL2-associated X protein	1.64	0.0257
NM_005912	Melanocortin 4 receptor	1.63	0.0102
NM_025069	Zinc finger protein 703	1.62	0.0105
NM_001077516	Solute carrier family 39 (zinc transporter), member 7	1.62	0.0146
NM_001384	DPH2 homolog	1.61	0.0181
NM_001005282	Olfactory receptor, family 5, subfamily M, member 8	1.56	0.0326
NM_000433	Neutrophil cytosolic factor 2	1.56	0.0087
NM_001849	Collagen, type VI, alpha 2	1.54	0.0395
NM_017726	Protein phosphatase 1, regulatory (inhibitor) subunit 14D	1.53	0.0179
NM_173611	Family with sequence similarity 98, member B	1.51	0.0256
NM_012401	Plexin B2	1.5	0.0040
NM_014598	**Suppressor of cytokine signaling 7**	**1.49**	**0.0393**
NM_207357	Hypothetical LOC339524	1.48	0.0139
NM_001100912	BEN domain containing 7	1.47	0.0388
XM_497642	**T cell-interacting, activating receptor on myeloid cells 1**	**1.47**	**0.0043**

These genes are the 25 most highly upregulated genes with known functions by LPS+LBP in HMGECs. Only 2 genes, in bold type, are associated with inflammation. Relative ratios were calculated by comparing the degree of gene expression in HMGECs treated with LPS+LBP, relative to vehicle.

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Table 11

LPS+LBP up-regulation of immune-related gene expression in HMGECs

Accession number	Gene	Ratio	P value
NM_002502	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	1.40	0.0176
NM_002994	Chemokine (C-X-C motif) ligand 5	1.37	0.0101
NM_001570	IL-1 receptor-associated kinase 2	1.26	0.0044
NM_007283	Monoglyceride lipase	1.25	0.0220
NM_000633	B-cell CLL/lymphoma 2	1.22	0.0089
NM_020529	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.18	0.0347
NM_022789	IL-25	1.14	0.0361
NM_032036	Interferon, alpha-inducible protein 27-like 2	1.13	0.0440
NM_002089	Chemokine (C-X-C motif) ligand 2	1.12	0.0395
NM_173843	IL-1 receptor antagonist	1.11	0.0205
	Toll-IL-1 receptor domain containing adaptor		
NM_052887	protein	1.10	0.0470
NM_002965	S100 calcium binding protein A9	1.08	0.0094
NM_001992	Coagulation factor II (thrombin) receptor	1.06	0.0250

Examples of up-regulated genes were selected from the innate immunity biological response ontology, as well as the list of all genes significantly influenced by LPS+LBP. Relative ratios were determining by comparing the degree of gene expression in HMGECs exposed to LPS+LBP, as compared to vehicle. Ratios for additional genes upregulated by LPS+LBP are listed in Table 10.

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Table 12

LPS+LBP influence on KEGG pathways in HMGECs

KEGG Pathway	LPS+LBP Genes ↑	Vehicle Genes ↑	LPS+LBP z-score	Vehicle z- score
Apoptosis	10	3	5.09	−0.05
Pathogenic Escherichia coli infection	6	0	3.8	−1.42
Neurotrophin signaling pathway	9	4	3.12	−0.23
B cell receptor signaling pathway	5	3	2.16	0.23

KEGG pathways were selected after the evaluation of non-log-transformed Illumina BeadChip data. The criterion for inclusion was a pathway linked to inflammation and a z-score > 2.0.

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Table 13

Influence of LPS+LBP on the expression of immune-related gene ontologies in HMGECs

Biological process ontology	LPS+LBP Genes ↑	Vehicle Genes ↑	LPS+LBP z-score	Vehicle z- score
Toll-like receptor 4 signaling pathway	8	3	4.6	0.12
Immune response-activating signal transduction	13	6	4.36	−0.17
Toll-like receptor 1 signaling pathway	7	2	4.35	−0.28
Toll-like receptor 2 signaling pathway	7	2	4.3	−0.3
Immune response-regulating signaling pathway	13	6	4.27	−0.23
Myd88-dependent toll-like receptor signaling pathway	7	2	4.12	−0.38
Negative regulation of type I interferon production	4	3	4.03	1.93
Toll signaling pathway	7	2	3.98	−0.44
Innate immune response- activating signal transduction	8	3	3.95	−0.21
Activation of innate immune response	8	3	3.88	−0.24
Antigen receptor-mediated signaling pathway	8	3	3.74	−0.31
T cell receptor signaling pathway	7	2	3.74	−0.55
Toll-like receptor 3 signaling pathway	6	2	3.69	−0.21
Activation of immune response	13	6	3.49	−0.71
Type I interferon production	5	4	3.47	1.6
Regulation of innate immune response	10	5	3.11	−0.42

Designated immune-related ontologies from the biological process category were selected after the analyses of non-log-transformed data. Criteria for inclusion were an ontology containing ≥ 5 genes and having a z-score > 3.0.